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HIGH LEVEL EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF THE KUNITZ-TYPE PROTEASE INHIBITOR DOMAIN OF PROTEASE NEXIN-2/AMYLOID β-PROTEIN PRECURSOR

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SUMMARY The protease inhibitor, protease nexin-2 (PN-2), is the secreted isoform of the Alzheimer's amyloid β-protein precursor (AβPP) that contains the Kunitz-type protease inhibitor (KPI) domain. Here we describe the use of the methylotrophic industrial yeast *Pichia pastoris* as a host system for the large scale production of the KPI domain of PN-2/AβPP. In addition to the 57 amino acid KPI domain, the expression product contained an additional four amino acid residues at its amino terminus that correspond to amino acids 285-288 of AβPP (Ponte et al. 1988 Nature 311:525-527). This expression system generated yields of greater than 1.0 gram of KPI domain per liter of fermentation media. The secreted 61 amino acid product was purified to homogeneity and biochemically characterized. Amino acid analysis and sequencing of the entire expressed KPI domain verified its integrity. Similar to native PN-2/AβPP, the purified KPI domain potently inhibited trypsin, chymotrypsin, and coagulation factor XIa. Although heparin augments the inhibition of factor XIa by native PN-2/AβPP it had no effect on the inhibition of factor XIa by expressed KPI domain suggesting that heparin binds to regions on native PN-2/AβPP outside of the protease inhibitory domain. This KPI domain expression product should be useful in studying the physiologic and pathophysiologic functions of PN-2/AβPP.

The amyloid β-protein (Aβ) is a 39-42 amino acid peptide that is deposited in senile plaques and in walls of cerebral blood vessels of patients with Alzheimer's disease, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis-Dutch type, and, to a lesser extent, in normal aged individuals (1-6). It is derived from abnormal proteolysis of its parent protein the amyloid β-protein precursor (AβPP) (7-10). AβPP can be translated from predominantly three alternatively spliced mRNA species to yield polypeptides of 695, 751 and 770 amino acids (11-

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ABBREVIATIONS: PN-2, protease nexin-2; AβPP, amyloid β-protein precursor; Aβ, amyloid β-protein; KPI, Kunitz-type protease inhibitor; αMF, alpha mating factor.

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13). The latter two species contain an additional insert which codes for a domain that is homologous to Kunitz-type serine protease inhibitors (KPI) (11-13). The KPI-containing isoforms of AβPP are analogous to the cell-secreted protease inhibitor, protease nexin-2 (PN-2) (14,15). Messenger RNA encoding the KPI-containing AβPP 751/770 isoforms are found in most tissues. In contrast, mRNA encoding the KPI-lacking AβPP 695 isoform is found primarily in brain. However, recent studies have indicated that the predominant isoform of AβPP protein in human brain contains the KPI domain (16).

Several studies have suggested potential physiologic functions for the KPI domain of PN-2/AβPP. Measurements of protease inhibition equilibrium constants revealed that PN-2/AβPP is a very potent inhibitor of intrinsic blood coagulation factor XIa (17,18). In this regard it is noteworthy that PN-2/AβPP is an abundant platelet α granule protein and is secreted by platelets that are activated by physiologic agonists (19-21). Together, these findings suggest that secreted platelet PN-2/AβPP may play a role in regulating the intrinsic blood coagulation cascade at sites of vascular injury where it is released by activated platelets (17,18,19,21). In addition to its potential role in regulating blood coagulation via platelets, PN-2/AβPP in brain may function as an intracerebral anticoagulant (22).

It has been suggested that the KPI domain of PN-2/AβPP may potentially be involved with the abnormal proteolysis of full-length AβPP leading to Aβ formation in Alzheimer's disease. Normal constitutive processing of full-length AβPP, resulting in the secretion of PN-2, is the result of proteolytic cleavage on the carboxy terminal side of Lys^{16} within the Aβ domain of AβPP (23,24). This site may be susceptible to cleavage by a "trypsin-like" enzyme. On the other hand, a methionine residue flanks the amino terminus of the Aβ domain in AβPP. This site may be susceptible to cleavage by a "chymotrypsin-like" enzyme. PN-2/AβPP is a potent inhibitor of "trypsin-like" enzymes and chymotrypsin (14,17). It is noteworthy that KPI-containing AβPP has been localized in neuritic plaques in Alzheimer's disease (25).

Here we report the high level expression, purification and biochemical characterization of the KPI domain. This will provide an invaluable tool to investigate the physiologic and pathophysiologic protease inhibitory functions of PN-2/AβPP.

METHODS

Materials. PN-2 was purified from human fibroblast culture medium using immunoaffinity chromatography (17). The cation exchange resin SP Spherodex LS was from IBF Biotechnics. The hydrophobic interaction resin Toyopearl butyl 650M was obtained from Tosohass. Human factor XIa was obtained from Enzyme Research Laboratories. Heparin was obtained from Calbiochem. Bovine trypsin, bovine chymotrypsin, *p*-nitrophenyl guanidinobenzoate, and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide were from Sigma. Pyro-Glu-Pro-Arg-*p*-nitroanilide was purchased from Helena Laboratories. Carbobenzoxy-Val-Gly-Arg-4-nitroanilide was obtained from Boehringer Mannheim.

Construction of the KPI expression vector. A synthetic DNA sequence encoding amino acid residues 285-345 of AβPP-751 (11) was constructed using eight overlapping oligonucleotides. The synthetic gene sequence was formulated by back translation of amino acids 285-345 using a codon frequency computer program [University of Wisconsin Genetics Group (UWGCG)] in combination with consensus data generated from known gene sequences of the yeast *Pichia pastoris*. The synthetic gene sequence was designed to contain a *Hind*III site at its 5'

end and both an *Eco*RI and *Bam*HI site at its 3' end. The oligonucleotides were synthesized on an ABI 380A DNA synthesizer by phosphoramidate chemistry and were purified by HPLC on a Dupont 8800 reverse phase Zorbax C8 column. The eight oligonucleotides were annealed overnight and then ligated. The annealed DNA was run on a 1.2% preparative agarose gel. Fragments of approximately 200 bp were isolated and resuspended in water.

DNA encoding a yeast-specific secretion signal was attached to the synthetic gene utilizing plasmid pAO203 which is a pUC18-based plasmid comprised of the *S. cerevisiae* α-mating-factor (αMF) prepro signal sequence, including the Lys-Arg-(Glu-Ala-Glu-Ala) processing sites, the GRF gene, and *P. pastoris* AOX1 promoter and terminator sequences (26). The annealed and purified synthetic KPI oligonucleotide (≈2 µl) and 100 ng of pAO203, previously digested with *Bam*HI and *Hind*III and purified on a 0.8% agarose gel, were ligated together in a standard reaction to generate plasmid KPI100. A ≈450 bp *Eco*RI fragment corresponding to the signal sequence plus the KPI region was sequenced using the USB Sequenase Version 2.0 kit. This analysis revealed the sequence was correct except for a one base deletion. Two successive rounds of *in vitro* site-directed mutagenesis (27) were performed to correct the single base pair deletion and to delete the DNA encoding the Glu-Ala-Glu-Ala spacer between the α-MF-Lys-Arg sequence and KPI encoding sequences. The nucleotide sequence of the final 450 bp *Eco*RI fragment was sequenced and verified to be correct. This fragment was ligated with *P. pastoris* expression vector, pA0815, which had been digested with *Eco*RI and treated with calf intestinal alkaline phosphatase. Plasmid with the insert in proper orientation yielded a 930 bp band upon digestion with *Xba*I and was designated pKPI200. In plasmid KPI200 the αMF prepro-KPI fusion gene is operably linked to the *P. pastoris* alcohol oxidase (AOX1) gene promoter and terminator regions for methanol-inducible expression of the fusion in *P. pastoris*. Plasmid KPI200 was transformed into *P. pastoris* and expression of the KPI domain was conducted by high cell-density fermentation as previously described (28).

Purification of the expressed KPI domain. The KPI domain was expressed in 15 liter batch fermentations. The cells were separated from the fermentation broth by centrifugation. The resulting 7.4 liters of cell-free broth were diluted to 30 liters with deionized water and adjusted to pH 2.8 by the addition of 37 ml of concentrated phosphoric acid. The acidified, diluted broth was then loaded onto a 700 ml column of SP Spherodex LS cation exchange resin at a flow rate of 400 ml/min. The column was then washed with 3.2 liters of 50 mM acetic acid and the adsorbed KPI domain was eluted with four liters of 50 mM ammonium acetate, pH 5.5. The KPI domain was collected in several fractions as the major absorption peak at 280 nm. Since the KPI domain elutes in high enough concentration to precipitate at the elution pH, the KPI-containing eluates were adjusted to pH 7.0 by the addition of sodium hydroxide to redissolve the KPI domain before the next chromatography step. The resulting solution, containing 3-7 grams of KPI domain, was made 12% (w/v) ammoniun sulfate and was loaded onto a 1 liter Toyopearl butyl 650M hydrophobic interaction column at a flow rate of 70 ml/min. The loaded column was then washed with 12% ammonium sulfate and eluted in a 4 liter 12% to 0% gradient, followed by continued elution with deionized water. Loading 3 grams of KPI domain resulted in optimum separation. In order to remove the ammonium sulfate for lyophylization, the purified KPI domain fractions were pooled, diluted with two volumes of deionized water and acidified to pH 2.8 by the addition of concentrated phosphoric acid. The KPI domain was then loaded onto the original 700 ml cation exchange column, washed with 4 liters of 50 mM acetic acid, and eluted with 50 mM ammonium acetate, pH 5.5. The KPI domain-containing eluate was then lyophylized.

Protease inhibition measurements. The active sites of trypsin were titrated by the method of Chase and Shaw (29) using the burst titrant *p*-nitrophenyl guanidinobenzoate. This trypsin was used to titrate the protease inhibitory activity of the purified KPI domain. Briefly, 10 nM trypsin was incubated with increasing concentrations of purified KPI domain in 100 µl of 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5 containing 0.1% bovine serum albumin in 96-well microtiter plates for 20 min at room temperature. The remaining trypsin activity was then measured by the addition of 50 µl of 0.5 mM carbobenzoxy-Val-Gly-Arg-4-nitroanilide and following the change in absorbance at 405 nm in a microtiter plate reader (Molecular Devices). Inhibition equilibrium constants for purified PN-2 and KPI domain and factor XIa, trypsin, and chymotrypsin were determined as previously described (17).

RESULTS AND DISCUSSION

The expression of the KPI domain of PN-2/AβPP in bacterial systems has been previously described (30,31). However, in one of these systems the KPI domain was expressed as a bacterial fusion protein (30), and only relatively low levels of expression (1 mg/l) were achieved in the other system (31). In the present report we describe the high level expression of the KPI domain in the methylotrophic yeast *P. pastoris*. This yeast expression system has several marked advantages over the previously described bacterial expression systems including high level of product expression (28,32,33), low level of endogenous protein secretion (33), and, importantly, expression of native product without any yeast-specific amino acid additions. Furthermore, the fermentation methods employed can easily be upscaled to produce large quantities of KPI domain.

Eight overlapping synthetic oligonucleotides were used to construct a synthetic gene which encodes the 57 amino acid KPI domain (residues 289-345) and an additional upstream four amino acids (residues 285-288) of AβPP (Fig. 1). DNA encoding the yeast-specific secretion signal of *S. cerevisiae* αMF was attached to the synthetic KPI gene. The recombinant plasmid, pKPI200

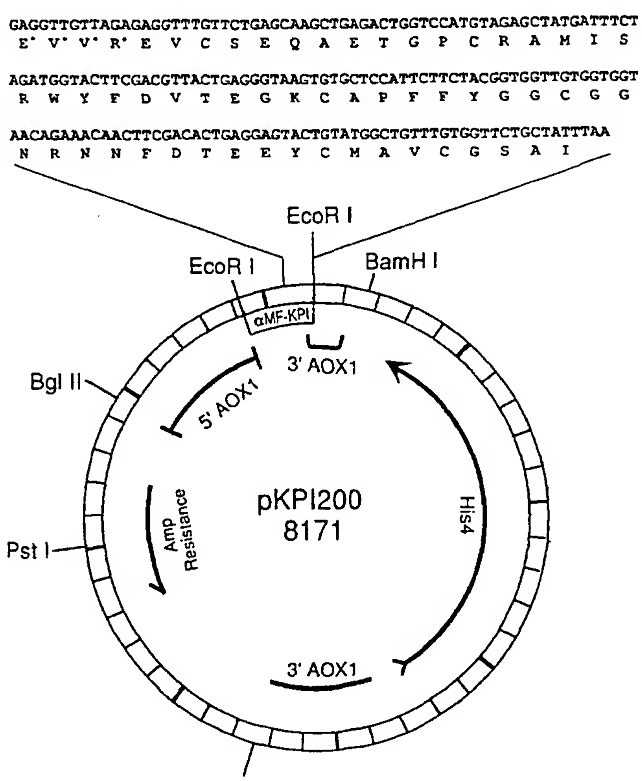

Fig. 1. Sequence of synthetic KPI gene and schematic of plasmid pKPI200. Shown is the nucleotide sequence of the synthetic KPI gene encoding the 61 amino acid expression product. The four amino terminal amino acids labeled with * correspond to amino acid residues 285-288 (11) of APP-751 that flank the KPI domain. The schematic shows the construct of plasmid pKPI200 with the synthetic KPI gene attached to DNA encoding the yeast αMF prepro secretion signal under control of the AOX1 promoter and terminator.

(Fig. 1) was transformed and expressed in the methylotrophic yeast *P. pastoris*. Transcription from the AOX1 promoter occurs at very high levels in *P. pastoris* cells grown in methanol and is particularly well suited for foreign gene expression (34,35). Transformed *P. pastoris* cells were grown in a fermenter in the presence of glycerol to accumulate biomass followed by feeding the cells with methanol to induce expression of the KPI domain. The secreted KPI domain comprised >80% of the protein in the fermentation media and achieved concentrations >1.0 gram/liter.

The expressed KPI domain was purified from the fermentation media following the procedures described in "Methods". The purified protein appeared homogeneous as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver stain analysis (Fig. 2) and reverse phase HPLC (data not shown). In addition, amino acid analysis and amino acid sequencing of the entire purified KPI domain revealed the integrity of the expressed product and showed that amino terminus of the protein was properly processed upon secretion (data not shown). The purified expressed KPI domain had a molecular mass of 6750 daltons. Titration experiments with the purified KPI domain revealed a 1:1 stoichiometry for trypsin inhibition (Fig. 3). Importantly, this demonstrated that the KPI domain was fully active and properly folded when expressed and secreted by the *Pichia* cells. The protease inhibitory properties of the expressed KPI domain were compared to that of purified PN-2. The protease inhibition equilibrium constants obtained for the inhibition of factor XIa, trypsin, and chymotrypsin by the purified KPI domain were nearly identical to those obtained for the inhibition of these proteases by purified PN-2 (Table 1) in agreement with previously reported measurements (17,18,30). In contrast to PN-2,

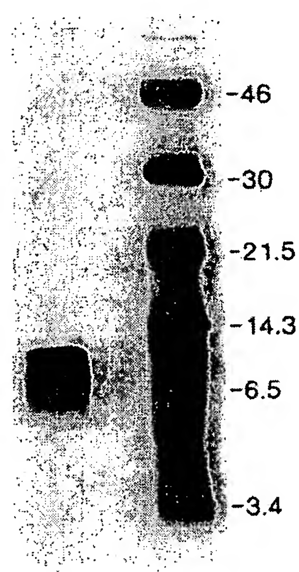

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver stain analysis of the purified KPI domain. ≈5 μg of purified KPI domain was electrophoresed on a 15% polyacrylamide gel according to Laemmli (36) and analyzed by silver staining. Lane 1, KPI domain; lane 2, molecular mass markers.

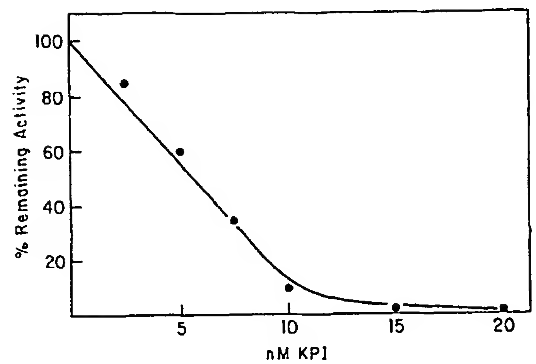

Fig. 3. Titration of the protease inhibitory activity of purified KPI domain with trypsin. Ten nanomolar trypsin was incubated with increasing concentrations of purified KPI domain and the residual trypsin activity was measured using the chromogenic substrate carbobenzoxy-Val-Gly-Arg-4-nitroanilide as described in "Methods".

however, the ability of the KPI domain to inhibit factor XIa was not enhanced by heparin. This indicates that the heparin binding domain of PN-2/AβPP resides outside the KPI domain of the protein. The heparin binding domain of the PN-2/AβPP may also serve to localize the protein and its protease inhibitory activity to specific sites *in vivo* (i.e. platelet and/or cell surfaces at sites of vascular injury).

In conclusion, the expression of native and fully active KPI domain in *P. pastoris* should provide an abundant source of an invaluable tool to probe the physiologic and potential pathophysiologic functions of this protease inhibitory domain of PN-2/AβPP. In addition, the high level of expression achieved in this system can readily provide very large quantities of the KPI domain for future use as a potential therapeutic agent.

TABLE 1. Protease inhibition constants for PN-2/AβPP and KPI domain

	K_i (M)	
Protease	PN-2/AβPP	KPI
Factor XIa + 10 U/ml heparin	$5.5 \pm 3.5 \times 10^{-11}$	$3.1 \pm 0.6 \times 10^{-10}$
Factor XIa	$4.0 \pm 0.8 \times 10^{-10}$	$4.5 \pm 1.8 \times 10^{-10}$
Trypsin	$8.3 \pm 2.2 \times 10^{-10}$	$3.1 \pm 1.2 \times 10^{-10}$
Chymotrypsin	$3.8 \pm 1.2 \times 10^{-9}$	$2.3 \pm 0.9 \times 10^{-9}$

The substrates and their final molarities are as follows: pyro-Glu-Pro-Arg-*p*-nitroanilide, 0.5 M (factor XIa); Carbobenzoxy-Val-Gly-Arg-4-nitroanilide, 0.5 M (trypsin); and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, 0.25 M (chymotrypsin). The protease inhibition equilibrium constants (Ki) were determined as previously described (17).

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